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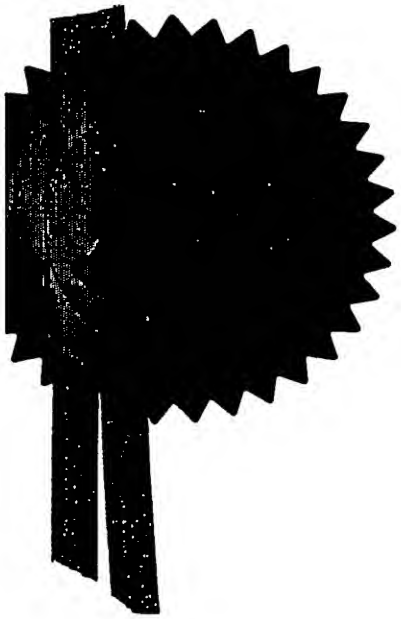

Signed Andrew Gerry

Dated 17 March 2005

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SMW/CP6206106

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

DANIOLABS LIMITED
7330 Cambridge Research Park
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Patents ADP number *(if you know it)*

08278921002

If the applicant is a corporate body, give the country/state of its incorporation

GB

4. Title of the invention

SCREENING METHODS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

MEWBURN ELLIS LLP
York House
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London WC2B 6HP

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~~109006~~ 8836884901

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Answer YES if:

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Continuation sheets of this form	0
Description	21 ✓
Claim(s)	4 ✓
Abstract	0
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Signature(s) Mewh Ellis Date 17 February 2004

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020 7240 4405 Seán M Walton

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SCREENING METHODS

The present invention relates to use of fish, in particular zebrafish, in screening for substances with an ophthalmological effect or biological effect on the brain or central nervous system and/or effect on a disease or disorder of the brain or central nervous system. It further relates to use of zebrafish in screening for substances that have a desired biological activity and which do not cross the blood brain barrier.

The invention is based in part on the inventors' finding that zebrafish have a blood brain barrier (BBB) and that this blood brain barrier becomes established at defined times. While all vertebrates have some form of BBB, this barrier is poorly characterised in lower vertebrates. While some clear differences have been identified in the properties of the BBB in teleosts and mammals, e.g. monoamines can cross the BBB of teleosts but not rodents (Khan and Deschaux, 1997) little is known about how the BBB differs in terms of either structure or function between vertebrate species. Furthermore, it is known that, in mammals, the BBB "tightens" throughout development and is only mature in post-natal animals (Ibiwoye et al., 1994; Wolburg and Lippoldt, 2002; Nag., 2003). One of the major benefits of the zebrafish as a model organism is the ability to perform screens on juvenile stages, but knowledge about the presence of a blood brain barrier in such organisms and developmental time of formation is not available in the art. The knowledge now provided for the first time herein has important technical application in design and execution of screens for substances that have desired biological effects in the brain or central nervous system, ophthalmologically and so on.

Brief Description of the Figures

Figure 1 is a schematic flowchart setting out screening in accordance with an embodiment of the present invention. Here, as elsewhere here, reference to "post day 5" is to 5 days post-fertilisation or later, i.e. following establishment of the blood brain barrier as determined herein.

Figure 2 is a histogram showing mean fluorescent intensity in the brain of Evans Blue injected fish and saline controls.

Figure 3 is a schematic flowchart incorporating the features of claim 1 and further illustrating use of results obtained in screens according to the present invention in assessing biological activities of test substances.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which makes pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process have considerable commercial importance and utility.

In various further aspects the present invention relates to screening and assay methods and means, and substances identified thereby.

More specifically, the present invention employs zebrafish in screening and assay methods for substances that are biologically

active in the brain or central nervous system (CNS) and/or exert an ophthalmological biological effect, e.g. in ameliorating one or more symptoms of a disease or disorder of the brain or central nervous system, wherein the screening or assay methodology takes into account knowledge (1) that zebrafish have a blood brain barrier and (2) that the blood brain barrier forms in zebrafish embryos at five days.

It should be noted that reference herein to establishment of a blood brain barrier at five days post fertilisation is a reference to establishment of a blood brain barrier sufficient to exclude molecules of comparable Mr to Evans Blue. For smaller molecules, effective blood brain barrier development may be excluded later. Thus, in all aspects and embodiments of the present invention, should it be determined that smaller molecules are able to pass the blood brain barrier at five days post fertilisation but are excluded from a later point in time, then that point in time (e.g. 6 days or 7 days post fertilisation) is to be substituted for reference to 5 days post fertilisation where the test substance is of such smaller size.

The present invention for example provides a method of screening for a substance with the desire effect wherein the method is substantially as set out in Figure 1. "Compound x" refers to a test substance.

The present invention provides a solution to technical problems associated with lack of knowledge in relation to the blood brain barrier.

In the absence of knowledge about formation of a blood brain barrier in zebrafish, false negative or false positive results may be obtained when searching for substances with an effect on the brain or central nervous system.

For example, if screening is performed after the blood brain barrier has formed (as determined by the present inventors) then a substance that could exert the desired biological effect if it were delivered to the brain but does not exert the desired biological effect when not delivered specifically to the brain because it does not cross the blood brain barrier will yield a false negative result. A person testing that substance without knowing that there is a blood brain barrier across which the substance cannot pass will discard the substance as not useful for achieving the desired biological effect, even though if the right delivery were used (e.g. directly into the brain or eye) the substance would in fact be useful for achieving that biological effect.

On the other hand, if such a search is performed using zebrafish prior to formation of the blood brain barrier (as determined by the present inventors), then a result may be obtained equivalent to a false positive result. For instance, a substance may exert the desired biological effect in the screen, but then later fail in adults because it is not able to cross the blood brain barrier. The substance may then be discarded as not useful, after some significant input of effort in the light of the initially positive result in the screen. Assessing ability to cross the blood brain barrier and, moreover, employing test fish at an appropriate stage of development, taking into account the

knowledge provided by the present invention, avoids such problems and is of great benefit in the art.

In accordance with aspects and embodiments of the present invention, applying knowledge of the existence and time of formation of the blood brain barrier in zebrafish, the ordinary skilled person will obtain better and more useful results with test substances. For instance, a substance known not to be able to cross the blood brain barrier may be tested for desired biological activity in a screen that is designed taking into account the presence or absence of a blood brain barrier in the fish employed in the screen. Fish embryos may be used prior to their formation of a blood brain barrier and substances found to have the desired biological effect. Additionally or alternatively, substances may be tested in fish in which a blood brain barrier has formed, thereby establishing not only ability to exert the desired biological activity but also ability to cross the blood brain barrier. An option is to deliver the substance directly to the brain in a fish in which the blood brain barrier is known to have formed, thereby testing its ability to exert the desired biological effect without limitation as to its ability to cross the blood brain barrier.

In further embodiments, the invention provides for screening methods for substances that exert a desired biological activity in the body but also an undesired biological activity on a target that exists in the brain, central nervous system and/or eye. Thus, a screening method may be employed to determine biological activity of a test substance and to determine ability or inability of the test substance to cross the blood brain barrier, e.g. by determining whether or not the substance appears or

appears to any significant extent within the brain, central nervous system or eye when not administered directly to these tissues. Knowledge about the existence of the blood brain barrier in zebrafish and time of its establishment allows for screening methods to be designed in which exclusion of substances from the brain, central nervous system and/or the eye by virtue of inability to cross the blood brain barrier can be determined, and employed for example in selection of lead compounds for further development as drugs.

The skilled person is well versed in design and implementation of biological screens and application of appropriate control experiments. The present invention extends to screens in zebrafish that take into account the existence of the blood brain barrier in zebrafish and the time of its formation.

In various aspects and embodiments the present invention provides the subject-matter set out in the claims below.

The invention is generally applicable to any of a variety of diseases and disorders, and a range of examples is specifically set out as follows: The following diseases are common disorders of the central nervous system. A common feature of all these disorders is that the cell to be targeted by a therapeutic lies behind a cellular barrier - the blood brain, or blood retinal barrier.

Retinal degenerations, including:

Macular degeneration

Retinitis pigmentosa

Ganglion cell degeneration (glaucoma)

Demyelingating disorders, including:
- Multiple sclerosis
- ADEM

Degenerative disorders, including:
- Parkinson's Disease
- Alzheimer's Disease
- Huntington's Disease
- Diseases with motor neuron inclusions
- Tauopathies
- Corticobasal degeneration

Neuropsychiatric disorders, including:
- Depression
- Bipolar disease
- Schizophrenia
- Anxiety
- Aggression
- Sexual dysfunction

Miscellaneous, including:
- Epilepsy
- Headache
- Pain
- Sleep disorders
- Satiety
- CNS / retinal malignancies

Rarer conditions of the CNS and retina can be found in any standard medical textbook.

The present invention provides for formulation of an algorithm to apply in design of screening assays, taking into account the existence of the blood brain barrier in zebrafish (as determined

by the present inventors) and the time of formation of the blood brain barrier, optionally also whether or not a test substance is known to be able or not to be able to penetrate the blood brain barrier. By means of an algorithmic approach, the skilled person determines which combination or combinations of experiments employing zebrafish of particular age, mode and site of delivery of test substance, nature of test substance, method of determining effect and so on are employed in the screening method. Furthermore, such an approach allows for interpretation of results obtained in different experiments, e.g. as illustrated in Figure 3.

The test zebrafish may have one or more symptoms or signs of a disease or disorder prior to use in screening for a substance with the desired activity.

The test substance may be administered with or prior to the administration of a substance which also induces one or more symptoms or signs of the disease or disease of interest.

Thus the invention is equally applicable to screening for substances which exert a biological effect that alters an activity or function in the central nervous system, brain or eye, whether normal or subject to a disease or disorder, as to screening for substances which exert a biological effect that is ameliorative of a sign or symptom of a disease or disorder, is therapeutic or is prophylactic.

The test organism is a zebrafish.

The zebrafish is an organism which combines many of the advantages of mammalian and invertebrate model systems. It is a vertebrate and thus more relevant in models of human disease than *Drosophila* or other invertebrates, but unlike other vertebrate models it can be used to perform genetic screens.

A number of peer reviewed papers highlight and validate the use of zebrafish as a species in which to model human disorders. [Dooley K and Zon LI (2000) Zebrafish: a model system for the study of human disease. Current Opinion in Genetics and Development 10 : 252-6 -Barut BA and Zon LI (2000) Realising the potential of Zebrafish as a model for human disease Physiological Genomics 13: 49-51 - Fishman MC (2001) Zebrafish: The Canonical Vertebrate. Science 294: 1290-1].

The inventors have appreciated that zebrafish offer the unique combination of invertebrate scalability and vertebrate modelling capabilities. They develop rapidly, with the basic body plan already having been laid out within 24 hours of fertilization. Moreover, their *ex-utero* development within a transparent capsule allows the easy *in vivo* visualisation of internal organs through a dissecting microscope. Many disease states can be modelled within the first week of life, at which time the embryos are only a few millimetres long and capable of living in 100 µl of fluid. This permits analysis of individual embryos in multi-channel format, such as 96 well plate format. This is particularly useful for drug screening, with many chemicals being arranged in 96 well plate format.

A population of zebrafish in a petri dish or a tank may be employed. A population of fish may be treated together, and may

be tested together, e.g. via addition of one or more or a combination of test substances to the water.

The zebrafish has a short maturation period of two to three months and is highly fecund, with a single pair of adults capable of producing 100 to 200 offspring per week. Both embryos and adults are small, embryos being a few mm and adults 2-3 cm long. They are cheap and easy to maintain. The ability to generate large numbers of offspring in a small place offers the potential of large scalability.

Zebrafish are thus a valid organism for screening for substances with biological activity in vertebrates, including mammals, including humans, which substances are useful *in vivo*, for example as therapeutics. The present invention extends the value of zebrafish screens by allowing for improved design of screens and improved usefulness of results obtained.

Following identification of a test substance with desired biological activity using a screening method in accordance with any aspect or embodiment of the present invention, the test substance may be formulated into a composition comprising at least one additional component, for example including a pharmaceutically acceptable vehicle, carrier or excipient.

In various further aspects, the present invention thus provides a pharmaceutical composition, medicament, drug or other composition comprising a substance that has the desired biological activity or exerts the desired biological effect, the use of such a material in a method of medical treatment, a method comprising administration of such a material to a patient, e.g. for

treatment (which may include preventative treatment) of a medical condition, use of such a material in the manufacture of a composition, medicament or drug for administration for such a purpose, e.g. for treatment of an ophthalmic disorder or disorder of the brain or the CNS, and a method of making a pharmaceutical composition comprising admixing such a material with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

One or more small molecules may be preferred therapeutics identified or obtained by means of the present invention. However, the invention may be used to identify appropriate targets for antibody mediated therapy, therapy mediated through gene targeting or protein targeting, or any of a variety of gene silencing techniques, including RNAi, antisense and morpholinos. The zebrafish may be treated with the test substance in a number of ways. For example, fish may be contacted with the test substance, it may be touched or rubbed on their surface or injected into them.

A further advantage of zebrafish is the fact they live in water. This makes administration of test substances easy as they may be added to water in which the fish are. Zebrafish readily absorb chemicals. The effective concentration of chemicals in the water often equates to the effective plasma concentration in mammals.

Different test substances may be added to each well of a multi-well plate, such as a 96 well plate, to identify that test substance exhibiting a beneficial or deleterious effect. There may be one or multiple fish in each well exposed to the test substance.

Zebrafish are also DMSO (dimethyl sulphoxide) tolerant. This is important as DMSO is used as a solvent to dissolve many drugs. The inventors have established that zebrafish can tolerate 1% DMSO. Thus, a candidate drug or other test substance may be dissolved in DMSO and administered to zebrafish by adding to the fish water to give a final concentration of DMSO of at least up to 1%. This is employed in various preferred aspects and embodiments of the present invention.

The same test substance may be added to different wells at a different concentration. For example, test substance 1 may be added to well A1 at a concentration of 1mM, to well A2 at a concentration of 100uM, to well A3 at a concentration of 10uM, to well A4 at a concentration of 1uM and to well A5 at a concentration of 0.1uM. Then test substance 2 to well B1 etc. The panel of test substances may be known drugs or new chemical entities.

Additionally, the test substances may be added in combination. For example, well A2 may contain test substance 1 and 2, well A3 test substance 1 and 3, well B2 test substance 2 and 3. Alternatively, every well may contain test substance x, with individual wells containing a panel of additional test substances.

In other options, a population of zebrafish in a petri dish or a tank may be employed and treated together, e.g. via addition of one or more or a combination of test substances in the water.

For delivery into the brain a delivery system may be employed, for example using a lipophilic delivery molecule, with the whole "trojan horse" being administered to the fish water. Alternatively, the test substance may be injected directly with the aid of a micromanipulator, into the CNS, for example into a ventricle, thus bypassing the BBB.

Thus, zebrafish enable the entire biological pathway of a vertebrate to be screened in a high-throughput fashion.

The present invention in certain aspects and embodiments provides for screening for and preferably identifying or obtaining a substance that provides a synergistic combination with another substance, or for screening for and preferably identifying or obtaining two or more substances that together provide an additive or synergistic combination. Clinical benefit is often derived from synergistic combinations of drugs. Use of a screening system in accordance with the present invention allows for identification of such synergistic combinations.

Thus, in certain embodiments the invention comprises treating the zebrafish, as discussed, with two or more substances, at least one of which is a test substance, and comparing the effect of the two or more substances in combination to determine the optimum effect (whether simultaneously or sequentially applied) on an aspect of behaviour or physiology with the effect of either or both of the two or more substances when applied individually or alone. Either all (or both) of the substances applied may each be a test substance, or one of the substances may be a drug known to have a beneficial effect in the disease that is the subject of the screen, or at least an effect in the test fish.

The invention thus provides for screening for and preferably identifying or obtaining a substance that provides an additive effect to a known drug or a synergistic effect with the known drug. It also provides for screening for and preferably identifying or obtaining a combination of two or more substances that provide a synergistic effect, compared with the effect of the two substances when employed individually or alone.

Add-on therapies are useful because it is difficult to conduct clinical trials in which an existing drug is withdrawn from a patient and replaced with a new drug. The patient is deprived of a drug which has at least got some proven efficacy and some confidence in its side-effect profile. Additionally, the patient will be vulnerable to their disease during the phases of withdrawal of the existing drug and build up of the test drug.

The test zebrafish may be mutated rather than a wild-type. It is then possible to assay for interacting effects, either beneficial synergistic effects, or deleterious effects, of the mutation plus the test substances. Alternatively, the analysis may be of the known therapeutic agent and the genetic mutation to discover either a new drug target of benefit in combination with the known drug, or a genetic marker of use in predicting which patients are most likely to benefit (or not benefit) from prescription of the known drug.

In another embodiment, a combination of potentially useful agents is administered to a test zebrafish having one or more symptoms of a disease or disorder, which may be generated through addition of an agent to the test fish, e.g. via addition to the fish water

or through expression or knock out of a gene, to assess whether the combination is more effective than either of the individual agents.

The present invention also provides for screening for and preferably identifying or obtaining a substance that ameliorates one or more side effects of an active substance, e.g. a therapeutically active substance. There are many drugs which have been discontinued in clinical trials, or are marketed but infrequently prescribed, not because they are not therapeutically effective, but because their side-effect profile is limiting. The side-effects may be relatively benign, but significant to the patient, such as renal damage (e.g. cyclosporin). It is desirable to allow the administration of such drugs, with proven beneficial effects, through the co-administration of an additional agent to improve the side-effect profile.

In accordance with the present invention, such agents are screened for in zebrafish in which administration of the active substance induces a side-effect or other phenotype reflective or indicative of a side-effect. Thus in embodiments of the invention, an active agent is administered to test zebrafish having one or more symptoms of an autoimmune disease and the side-effect of other phenotype is assessed for such fish when subjected to one or more test substances. This does not require *a priori* knowledge of action of the co-administered agent. In other embodiments, agents that achieve the desired therapeutic effect with a reduction of side-effects can be screened for and preferably identified or obtained by means of assessment of disease phenotype and side-effect phenotype. As with other aspects and embodiments of the present invention, this may

involve co-administration of a primary compound together with either a battery of candidate substances, or together with randomly induced genetic mutation. With the latter approach, i.e. mutation, subsequent steps are needed to identify the appropriate co-therapeutic following identification of fish with a mutation that provides an ameliorative effect.

A diverse library of drug-like compounds, such as the LOPAC library (Sigma) may be used, or the Chembridge PHARMACOphore diverse combinatorial library. Other targeted libraries against particular targets classes may be used, such as ion channel libraries or G protein libraries.

Following identification of an active substance using a screen in accordance with the present invention, the substance may be used in a method of medical treatment of the present invention, with administration preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials

should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The application of a test substance to zebrafish in a screening method may be as follows, in accordance with embodiments of the present invention:

1. A test substance is added to the test fish either prior to the appearance of the disease state, at the time of induction of the disease state, or after the induction of the disease state. The first two situations are more likely to identify a prophylactic chemical, the latter a drug which reverts the disease state back to normal. The test substance may be a chemical and may be a random chemical administered in a high-throughput fashion to fish in 96 well plate format, or a selected chemical administered to a clutch of fish in a Petri dish.

2. The fish is then screened for deviation from the initial disease state.

The following additional steps are highly desirable in screening, and their use is provided by the present invention in preferred embodiments:

Rather than add a single chemical, a combination of chemicals is added. For instance, a known therapeutic agent may be administered to all fish at a dose at which a further beneficial effect could still be detected. A random chemical library is then added to fish and an incremental effect screened for.

1. Induce an autoimmune disease state by the administration of antibodies to fish.

2. Administer drug 1 to row 1, drug 2 to row 2, etc.

3. Administer drug 1 to column 1, drug 2 to column 2 etc.

4. Compare the immunosuppressive effect of all wells with that of the drugs when given alone.

A further embodiment of the above allows for detection of augmentation of a particular drug through a particular mutation, as follows:

1. Induce genetic mutation through any of the above.

2. Induce disease state through any of the above.

3. Administer test chemical.

4. Assess whether the combination of the mutation plus chemical is greater than either alone.

5. The mutated gene is then used as a beneficial target, as described above.

A further embodiment of the invention allows identification of genetic factors which help determine the appropriateness of a particular therapeutic agent for a given patient. If the mutation augments the effect of the drug, that mutation is searched for in human homologues. Patients with this mutation should be preferentially prescribed the drug. If the mutation leads to a deleterious effect or lack of effect, then patients should avoid this drug.

EXPERIMENTAL

Evans Blue dye (961 Da) was used to investigate the presence of BBB in zebrafish larvae. Evans Blue is used in rodent studies of BBB and is excluded from the brain if the BBB is intact. In rodents, Evans Blue leakage is observed following brain trauma.

1) 2% Evans Blue in 0.9% saline was injected into pericardial sac of larvae from 2 days post-fertilisation (d.p.f.) to 1 month old. 0.9% saline was injected as a control.

2) The larvae were left to develop for 6 hours to allow the dye to penetrate from bloodstream into tissues.

3) The larvae were viewed under fluorescent dissecting scope (TRITC filter) to ensure that dye was present in blood vessels.

4) Larvae were anaesthetised and fixed in 4% PFA at 4 °C overnight.

5) They were embedded in O.C.T. (Optimal Cutting Temperature compound) and 10 µm frozen sections were taken in parasaggital plane.

6) Midline sections were viewed on fluorescent microscope (TRITC filter) at x40 magnification. Images were taken of Evans Blue injected and saline injected samples.

7) Fluorescent intensity was measured in 3 regions of control and Evans Blue injected samples using AnalySis software. Mean fluorescent intensity was calculated for each age group.

8) Mean fluorescent intensity in Evans Blue injected vs saline controls was compared by means of a histogram (Figure 2).

Results

High level of fluorescence in the brain at 3 d.p.f. provided indication that Evans Blue crossed into the brain and the BBB was not established. By 5 d.p.f., brain fluorescence in Evans Blue injected samples was no different from saline injected samples, demonstrating that Evans Blue was excluded and providing indication that the BBB had developed by 5 d.p.f.

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CLAIMS

1. A screening method to obtain a substance with biological activity in the central nervous system (CNS), brain or eye, the method comprising:

administering a test substance to test zebrafish

determining effect of the test substance on an activity or function of the central nervous system, brain or eye, or effect of the test substance on a symptom or a disease or disorder of the central nervous system, brain or eye of the test fish,

thereby identifying a substance with said biological activity;

wherein:

the test substance is known to penetrate the blood brain barrier and the method is performed on zebrafish of any age;

or the test substance is known not to penetrate the blood brain barrier and the method is performed on zebrafish of age less than five days post-fertilisation and/or is performed on zebrafish of age at least five days post-fertilisation and comprises direct delivery of the test substance to bypass the blood brain barrier;

or, without knowing whether or not the test substance is able to penetrate the blood brain barrier, the method is performed on zebrafish of age at least five days post-fertilisation with direct delivery of the test substance to bypass the blood brain barrier.

2. A method according to claim 1 wherein said direct delivery comprises injection into the brain or eye.

3. A screening method to obtain a substance with biological activity in the central nervous system (CNS), brain or eye, the method comprising:

administering a test substance to test zebrafish fish,

determining effect of the test substance on an activity or function of the central nervous system, brain or eye, or effect of the test substance on a symptom or a disease or disorder of the central nervous system, brain or eye of the test fish,

thereby identifying a substance with said biological activity;

wherein the method is performed on zebrafish of age less than five days post-fertilisation and is performed on zebrafish of age at least five days post-fertilisation without direct delivery of the test substance to bypass the blood brain barrier, whereby a substance which has said biological activity and has ability to penetrate the blood brain barrier is obtained.

4. A method for obtaining a substance with biological activity in the central nervous system (CNS), brain or eye, the method comprising a screening assay conducted by administering a test substance to test zebrafish whereby a substance with said biological activity is obtained, wherein design of the screening assay employs an algorithm formulated to take into account the presence of and time of formation of a blood brain barrier in the zebrafish.

5. A method according to claim 4 wherein the algorithm is further formulated to take into account whether or not the test substance crosses the blood brain barrier.

6. A screening method for a substance with biological activity in the central nervous system (CNS), brain or eye substantially as set out in Figure 1.

7. A method according to any one of claims 1 to 6 further comprising formulating the obtained substance with said biological activity into a composition comprising at least one additional component.

8. A method according to claim 7 wherein the composition comprises a pharmaceutically acceptable vehicle, carrier or excipient.

9. Use of the existence of and time of formation of a blood brain barrier in a zebrafish in the design of a screening assay for a substance with biological activity in the central nervous system (CNS), brain or eye.

10. A screening method to obtain a substance with biological activity, which substance does not pass the blood brain barrier, the method comprising:

administering a test substance to test zebrafish

determining effect of the test substance on an activity or function in the zebrafish or effect of the test substance on a symptom or a disease or disorder in the test fish,

thereby identifying a substance with said biological activity,

wherein the method further comprises determining ability or inability of the substance with said biological activity to cross the blood brain barrier in test zebrafish of age at least five days post-fertilisation, thereby identifying a substance with

said biological activity and which does not cross the blood brain barrier.

11. A method according to claim 10 comprising identifying the substance with said biological activity in a test zebrafish of age less than five days post-fertilisation.

12. A method according to claim 10 or claim 11 further comprising formulating the obtained substance with said biological activity into a composition comprising at least one additional component.

13. A method according to claim 12 wherein the composition comprises a pharmaceutically acceptable vehicle, carrier or excipient.

14. Use of the existence of and time of formation of a blood brain barrier in a zebrafish in the design of a screening assay for a substance with biological activity and which does not cross the blood brain barrier.

FIGURE 1

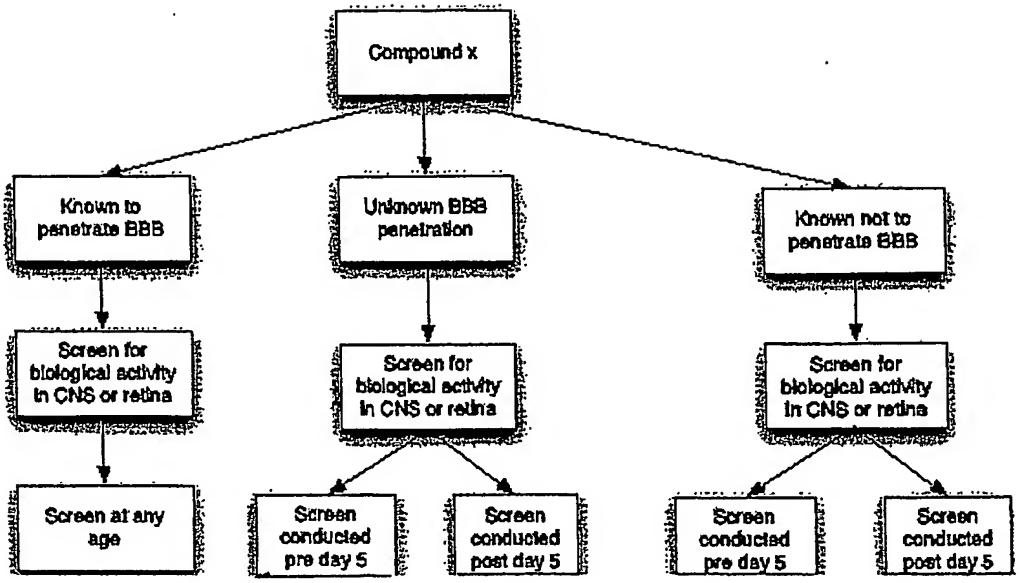


FIGURE 2

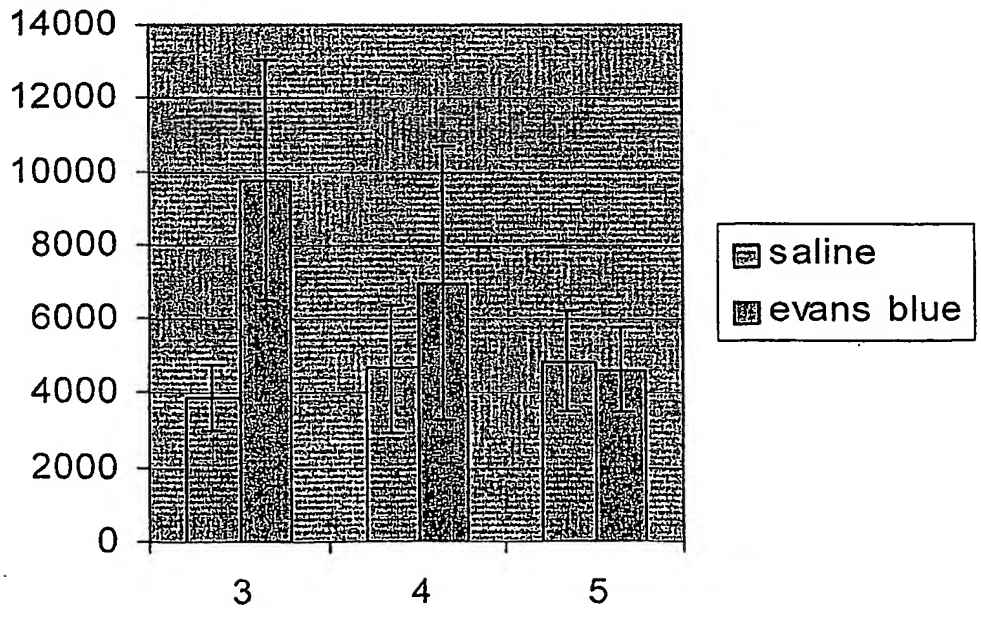
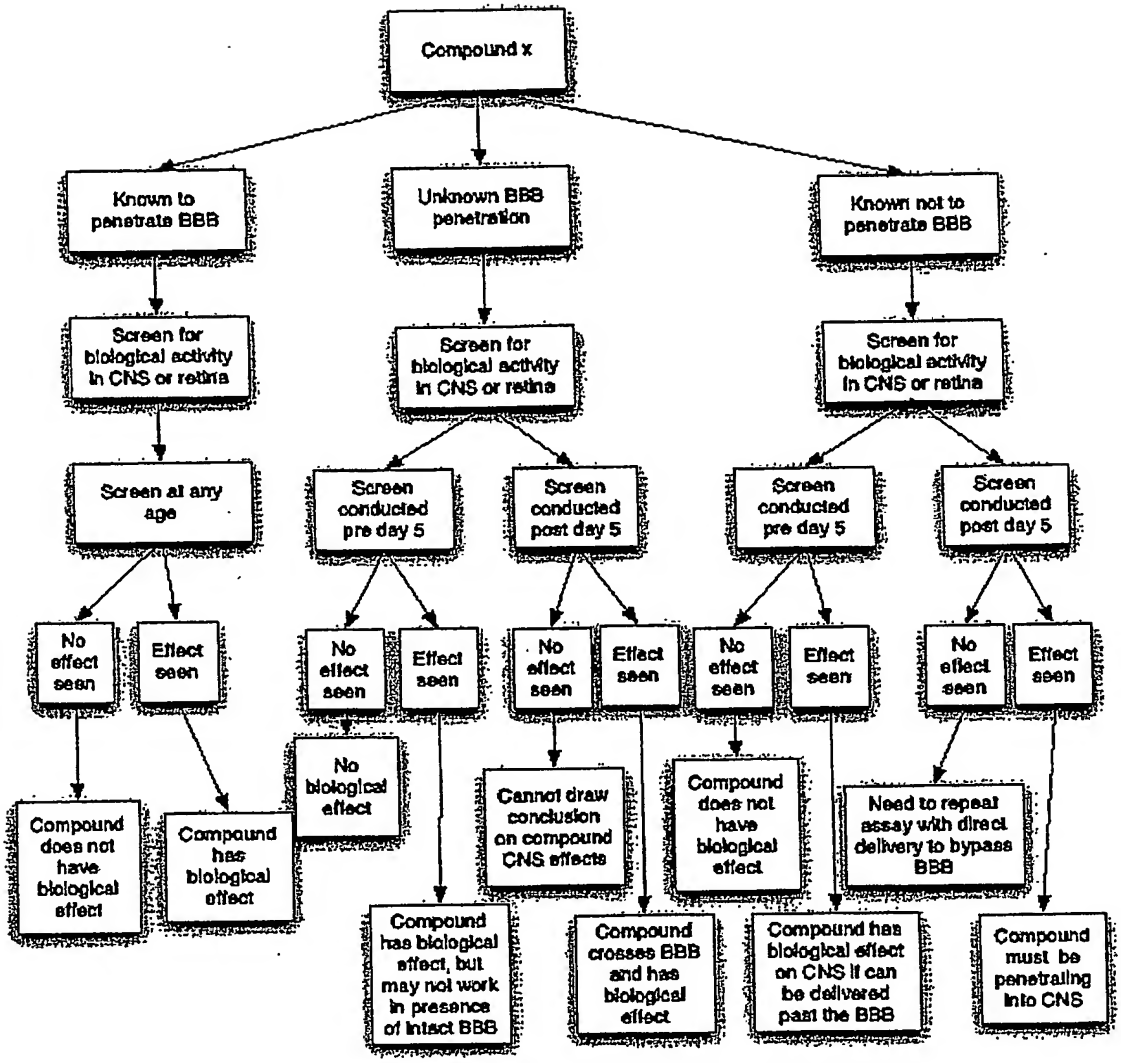


FIGURE 3


Compound x
Known to penetrate BBB
Unknown BBB penetration
Known not to penetrate BBB
Screen for biological activity in CNS or retina
Screen for biological activity in CNS or retina
Screen for biological activity in CNS or retina
Screen at any age
Screen conducted pre day 5
Screen conducted post day 5
Screen conducted pre day 5
Screen conducted post day 5
No effect seen
Effect seen
No effect seen
Effect seen
No effect seen
Effect seen
No effect seen
Effect seen
No effect seen
Effect seen
Compound does not have biological effect
Compound has biological effect
No biological effect
Cannot draw conclusion on compound CNS effects
Compound does not have biological effect
Need to repeat assay with direct delivery to bypass BBB
Compound has biological effect, but may not work in presence of intact BBB
Compound crosses BBB and has biological effect
Compound has biological effect on CNS if can be delivered past the BBB
Compound must be penetrating into CNS

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